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Comparative study on production of the alkaline protease enzyme from free and immobilized mycelia of *Aspergillus Niger* and *Aspergillus Flavus*

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ABSTRACT

Alkaline protease is an extracellular enzyme usually excreted into the fermentation medium during growth (especially at sporulation). Alkaline proteases are produced by various bacteria such as *Bacillus*, *Pseudomonas* etc. and by fungi such as *Aspergilllus niger*, *Aspergillus flavus* and Aspergillus *oryzae* etc. Alkaline proteases have found a wide application in several industrial processes such as an additive to detergents, in bating of hides and skins in leather industries. Alkaline protease holds more than 50% of the total enzyme market. It removes protein stains such as grass, blood, egg, milk and human sweat. As a result of the combined effect of surfactants and enzymes, stubborn stains can be removed from fibres. The immobilization procedure was adapted at industrial level to reduce the production time and production costs by recycling or reuse of the immobilized mycelia. Immobilised mycelia performed better over a wide range of temperature, pH and incubation period compared to free fungal mycelia. The mycelial mats were removed and inoculum was inoculated in the modified Reese's medium and the crude extract thus obtained was tested for protein estimation by Lowry's method. For immobilization sodium alginate matrix is used. *Aspergillus niger* was found to produce more amount of protease than *Aspergillus flavus*. Immobilized mycelia were found to withstand more environmental fluctuations. Immobilized mycelia can be recycled and is found to produce more protease when compared to free mycelia. Enzyme activity is determined by various enzymatic assays such as Protein agar plate assay and spectrophotometry.

Key words: Alkaline protease; *Aspergillus* sp; Reese medium; Immobilization; Sodium alginate; Lowry's method; Enzyme assays.

Abbreviations: SDA - Sabouraud's dextrose agar; LCBS – Lactophenol cotton blue stain.

DISCOVERY SCIENTIFIC SOCIETY

1. INTRODUCTION

Alkaline protease is an extracellular enzyme usually excreted into the fermentation medium during growth (especially at sporulation). Alkaline proteases are produced by various bacteria such as *Bacillus*, *Pseudomonas* etc. (Nehra et al., 1998; Rippon, 1988; Hanzi et al., 1993; Guravaiah et al., 2012), and by fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus oryzae* etc (Phadatare et al., 1993). Alkaline protease holds more than 50% of the total enzyme market. Alkaline proteases hydrolyse proteins and break them down into more soluble polypeptides or free amino acids. At present, cell immobilization technology is often studied for its potential to improve fermentation, increase production and bioremediation (Hagerman et al., 1985: Rosevear, 1984; Illanjiam & Arunachalam, 2021). There are three major techniques to be used for cell immobilization. They are entrapment, adsorption and coupling. The immobilization techniques which most resemble the circumstances, in which cells find themselves in nature, are their entrapment within gels and adsorption to surfaces (Lowry et al., 1951). For entrapment gels like carrageenan, sodium-alginate, agar-agar etc are used. Immobilizing mycelia in agar-agar, carrageenan and alginate are more beneficial as these compounds are non-toxic, easily available and cheap. Many microorganisms like Bacillus, *Aspergillus* spp are entrapped in these compounds (Kierstan and Bucke, 1977; Barett, 1995). These immobilized cells are used at industrial level for increasing the quantity and quality of the product at commercial level in the market.

2. MATERIALS AND METHODS

2.1. Collection of soil samples

A total of 5 soil samples from different locations were collected in sterile petriplates and covered with sterile polyethene bags. They were collected from locations in the (Table 1).

Figure 1

Mixed fungal colonies in primary isolation

Table 1 Soil samples from different locations

S. No.	Location	Type of soil
1.	Garden soil	Surface soil
2.	Soil from waste dumping area	Surface soil
3.	Soil from college	Subsurface soil
4.	Soil from the road	Subsurface soil
5.	Sandy soil from the Barren land	Subsurface soil

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2.2. Processing of Samples

2.2.1. Preparation of the Master Dilution

1 gm. of different soil samples were weighed and added aseptically in sterile 100ml distilled water. To form even soil suspension, samples were mixed with the help of glass rod or rotary shaker. This soil suspension is known as master dilution having dilution factor of 10^{-2}. Master dilution is further used for performing serial dilutions.

2.2.2 Primary Isolation

1ml from 5 different master dilutions were taken and serially transferred to tubes having 9 ml of sterile distilled water (Fig.1). Serial dilutions were carried up to 10^{-9} dilution. 1 ml of the suspension was discarded from the 10^{-9} dilution. 10^{-1}, 10^{-2} and 10^{-3} dilutions were taken from these dilutions for spreading on agar plates. From the above selected dilutions, 0.1ml of the suspension was taken with the sterile pipettes and poured at the centre of the Sabouraud dextrose agar (SDA) plates. With sterile L-rod the suspension was spread evenly over the SDA plates. Plates were incubated at room temperature till fungal growth was attained (approximately 3-4 days).

2.2.3 Macroscopic Examination

Colony morphology was observed (Fig.2 & 3).

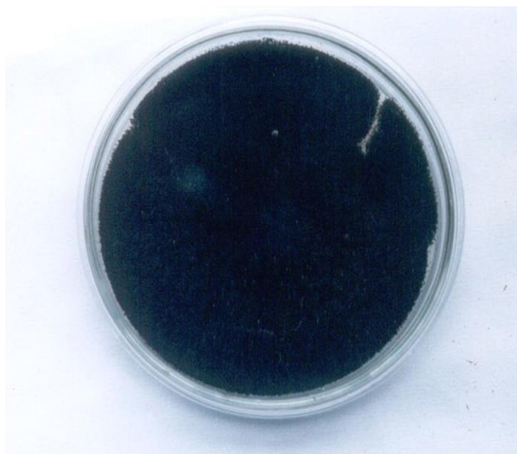

Figure 2
Secondary isolation of Aspergillus niger

Figure 3
Secondary isolation of Aspergillus flavus

2.2.4 Microscopic Examination of Isolated Fungus

Many different types of fungal colonies were isolated, from the soil sample, which was taken from the waste dumping area, belonging to surface level. Lactophenol cotton blue staining was performed for fungal identification (Fig.4 & 5).

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2.2.5. Lactophenol Cotton Blue Staining

Sterile, grease free slides were taken. A drop of lactophenol cotton blue stain (LCBS) was kept at the centre of the slides. A loopful of mycelia along with spores were taken and kept on the drop of LCBS. The mycelia were separated by teasing with the help of sterile needles. The slides were covered properly using coverslips. Slides were observed under 45x objective of the light microscope.

2.3. Secondary Isolation

The suspected *Aspergillus* species were taken for further secondary isolation, to obtain pure culture isolates.

2.3.1. Pure culture isolation

The suspected colony of *Aspergillus niger* and *Aspergillus flavus* were taken (Fig.2 & 3). From that colony, using sterile teasing needles, spores of *Aspergillus* spp were taken. The selected spores were inoculated at the centre of the SDA and Rose bengal chloramphenicol agar. The plates were incubated at room temperature, till the growth was attained.

2.4. Confirmation of pure culture isolates

This is done in 2 steps

a) Macroscopic confirmation

Colony morphology was observed (Table 2).

b) Microscopic confirmation

Pure culture isolates were confirmed with the help of lactophenol cotton blue staining and compared with Ainsworth classification (Fig.4 & 5). These pure culture isolates were inoculated in minimal SDA. Lactophenol cotton blue staining was performed (Table 3).

Table 2 Colony morphology of microorganism

S. NO.	MICROORGANISM	COLONY MORPHOLOGY
1.	*Aspergillus niger*	Wooly, at first white to yellow, then turning black-Reverse white to yellow.
2.	*Aspergillus flavus*	Velvety, yellow to green or brown. Reverse goldish red brown.

Table 3 Microscopic appearance of microorganism

S. NO.	MICROORGANISM	MICROSCOPIC APPEARANCE	
		CONIODIOPHORES	PHIALIDES
1.	*Aspergillus niger*	Long and smooth	Biseriate, cover entire vesicle, form “radiate” head.
2.	*Aspergillus flavus*	Variable length, Rough, pitted, spiny	Uniseriate and biseriate, cover entire vesicle, point out in all directions

2.5. Enrichment of *Aspergillus* species for protease production

Modified Reese’s medium in sterile 100ml Erlenmeyer flask was prepared and mixed well. The pH of the Reese’s medium should be adjusted to 9.0 before sterilization. The medium was sterilized in autoclave at 121°C for 15 mins at 15 lbs. 1ml of spore suspension was inoculated into the medium. The medium was then incubated at 40°C, till the proper growth was attained.

2.6. Preparation of immobilization matrix

Enriched fungal mycelia/spores from the enrichment medium were taken for immobilization. 4 gms of sodium alginate and 1.5 gms of agar was taken and added in 50 ml of distilled water and autoclaved at 15 lbs at 121°C for 5 mins. This solution was cooled to 40-45°C before use.

2.7. Immobilization of fungal mycelia /spores

Sterile 50ml distilled water was taken for making fungal suspension. This fungal suspension was added in the Na-alginate matrix and mixed properly. The suspension was pumped into cold calcium chloride solution. The alginate was then leached out with 0.05M potassium phosphate buffer (pH 7.5) and the immobilized mycelia/spores (25 gms) were added to the production medium.

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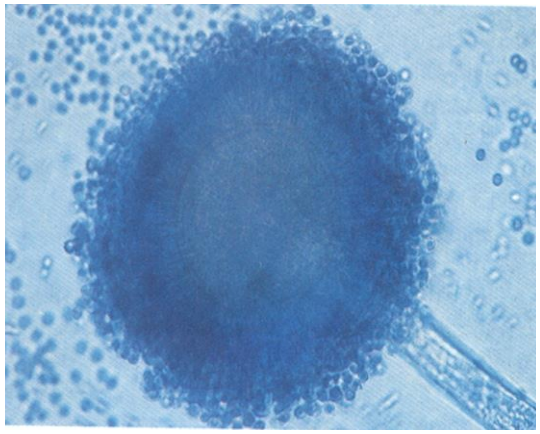

Figure 4
Microscopic observation of Aspergillus niger

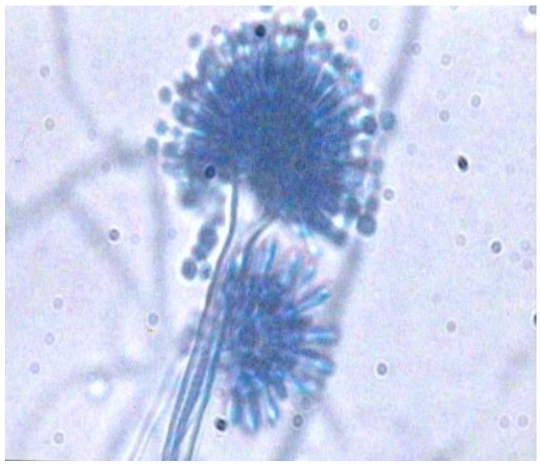

Figure 5
Microscopic observation of Aspergillus flavus

2.8. Alkaline protease production from immobilized Mycelia

Immobilized mycelium in Na - alginate matrix was added to 30ml of the medium broth in 100ml Erlenmeyer flask (Fig.8 & 9). Medium broth was then incubated at 35°C for 48 hrs on a rotary shaker. Culture medium was filtered with the help of Whatman filter paper and used as a crude source of enzyme.

2.9. Alkaline protease production from free mycelia

30 ml of modified Reese's medium (pH 8.5) was prepared in Erlenmeyer flask. Modified Reese's medium was then inoculated with 1.0 ml spore suspension. Modified Reese's medium was then incubated at 35ºC for 72 hours on a rotary shaker. The cultured medium was centrifuged at 10,000 rpm for 10 minutes and supernatant was used as a crude source of enzyme.

2.10. Alkaline protease enzyme assay

2.10.1. Determination of enzymatic activity (Qualitative analysis)

Casein agar was prepared in a sterile conical flask and autoclaved at 15 lbs/in² for 10 minutes at 121ºC. The supernatant and filtrate obtained were inoculated in three different ways in casein agar. The sterile Whatman filter paper discs were dipped in the supernatant/filtrate separately and kept in the center of the medium. With the gel puncture, wells were created in the center of the medium and with sterile Pasteur pipette; the wells were filled with the supernatant/filtrate. With inoculation loop, supernatants/filtrate was streaked on the medium separately. The plates were incubated for 24-48 hours. After incubation, zone of hydrolysis was observed (Fig.10 & 11).

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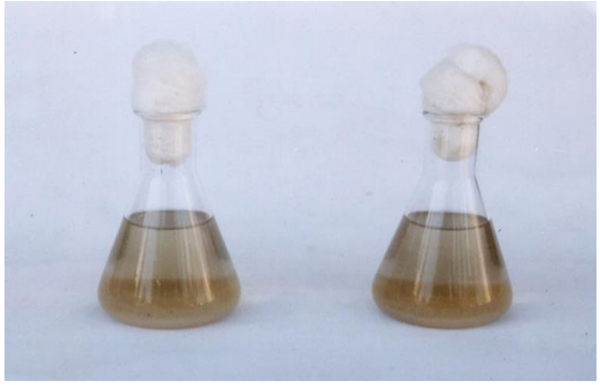

Figure 6

Production of alkaline protease from free mycelia before fermentation

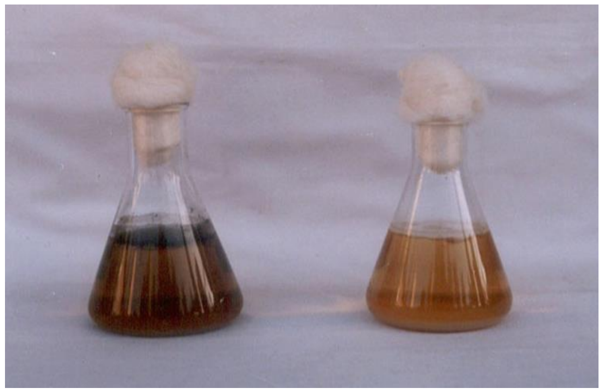

Figure 7

Production of alkaline protease from free mycelia after fermentation

Figure 8

Production of alkaline protease from immobilized mycelia before fermentation

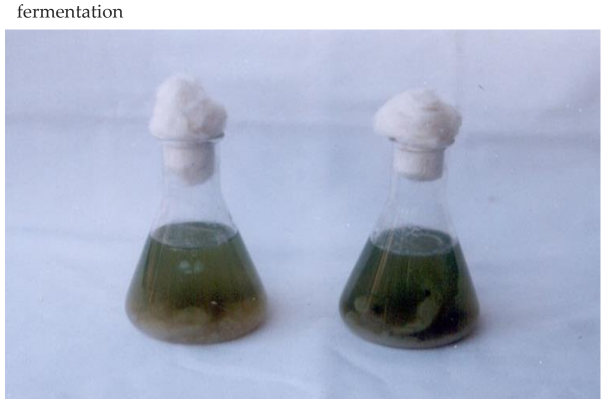

Figure 9

Production of alkaline protease from immobilized mycelia after fermentation

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Figure 10

Alkaline protease activity of Aspergillus niger

Figure 11

Alkaline protease activity of Aspergillus flavus

2.10.2. Estimation of protein (Quantitative analysis)

The amount of protein present in the crude filtrate/supernatant was determined by the Lowry's (1951) method.

Principle

The basis of the method is the reaction of protein with copper under alkaline conditions and the phosphomolybdic phosphotungstic acid reduction to heteroplymolybdenum blue by the copper catalysed oxidation of aromatic aminoacid, resulting in a blue colour which is measured at 660 nm. Phenols are capable of reducing molybdenum in complex of phosphomolybdo tungstenic acid.

Preparation- Protein stock solution

Bovine serum albumin of 50 mg/ml was weighed accurately and dissolved in distilled water and made upto 50 ml in a standard flask.

Procedure

Working standard of 0.2, 0.4, 0.6, 0.8 and 1 ml pipetted out into a series of test tubes. The volume was made upto 1 ml in all the test tubes with distilled water. 0.5 ml of supernatant / filtrate was mixed with 0.5 of distilled water. A test tube with 1 ml of water was served as the blank.To the diluted suspension, 5 ml of alkaline copper tartarate solution was added, mixed well and incubated at room temperature for 10 minutes. 0.5 ml of Folin's reagent was added, mixed and incubated at room temperature in the dark for 30 minutes. A blue colour was developed and the readings were taken at 660 nm. Using the readings standard graph was plotted. Optical density value of the supernatant was compared with the standard graph and the protein content (protease) of the supernatant/filtrate was estimated.

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3. RESULTS AND DISCUSSION

3.1. Macroscopic observation

Aspergillus flavus and Aspergillus niger were observed with different morphology on SDA & rose bengal chloremphenocol agar slants and plates (Table 2).

3.2. Microscopic observation

Less than 10x and 45x the microscopic appearance was observed and recorded (Table 3).

3.3. Effect of incubation period

Aspergillus niger produced more alkaline protease enzyme after 72 hrs in immobilized condition when compared to the free mycelia and Aspergillus flavus (Table 4 and Figs. 12 & 13).

Table 4 Effect of Incubation Period

Incubation Period (hrs)	Enzyme Estimated(μg/ml)			
	Production by free mycelia		Immobilised on Na-alginate	
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*
24	0.506	0.401	0.550	0.421
48	0.520	0.450	0.600	0.435
72	0.492	0.356	0.793	0.482

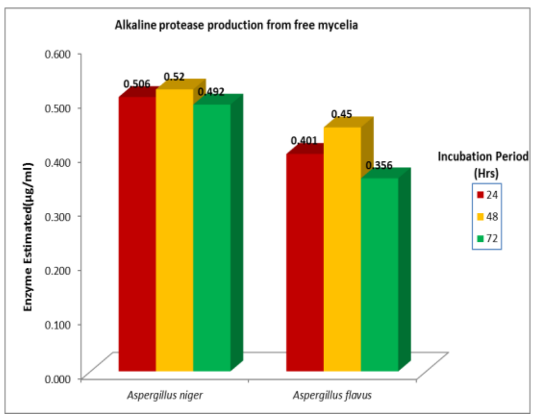

Figure 12

Effect of Incubation Period (free mycelia)

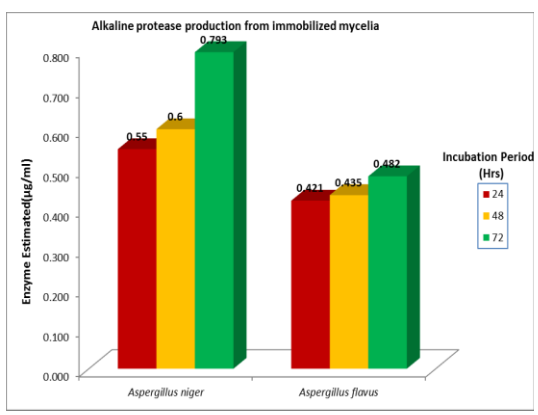

Figure 13

Effect of Incubation Period (immobilized mycelia)

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3.4. Effect of Temperature

In this environmental condition, Aspergillus niger produced more alkaline protease enzyme at 35℃ and Aspergillus flavus produce at 30oC in immobilized condition. When compared to the free mycelia Immobilized mycelia produce more alkaline protease enzyme. Among Both fungi Aspergillus niger produces more amount of enzyme (Table 5 and Figs. 14 & 15).

Table 5 Effect of Temperature

Temperature ºC	Enzyme Estimated(μg/ml)			
	Production by free mycelia		Immobilised on Na-alginate	
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*
30	0.401	0.435	0.529	0.485
35	0.609	0.435	0.790	0.488
40	0.300	0.235	0.800	0.270

3.5. Effect of pH

Immobilized Aspergillus niger produces the enzyme in high quantity at 8.5pH, whereas the Aspergillus flavus produces at 9.0pH (Table 6 and Figs. 16 & 17).

Table 6 Effect of pH

pH	Enzyme Estimated(μg/ml)			
	Production by free mycelia		Immobilised on Na-alginate	
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*
6.3	0.453	0.350	0.275	0.223
7.0	0.200	0.330	0.500	0.424
8.5	0.600	0.495	0.790	0.480
9.0	0.335	0.300	0.454	0.488

3.6. Effect of recycling on alkaline protease enzyme by free and immobilized mycelia of *Aspergillus Sp*.

Aspergillus niger and *Aspergillus flavus* produces more alkaline protease enzyme in immobilized condition after 1 & 2 cycles of reuse also, whereas the production of enzyme decreases after 1-cycle by free mycelia of Aspergillus sp (Table 7 and Figs. 18 & 19).

Table 7 Effect of recycling on alkaline protease enzyme by free and immobilized mycelia of Aspergillus sp.

Reuse Cycles	Enzyme Estimated(μg/ml)			
	Production by free mycelia		Immobilised on Na-alginate	
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*
1	0.388	0.424	0.4	0.387
2	0.402	0.3	0.695	0.65
3	0.2	0.198	0.5	0.477

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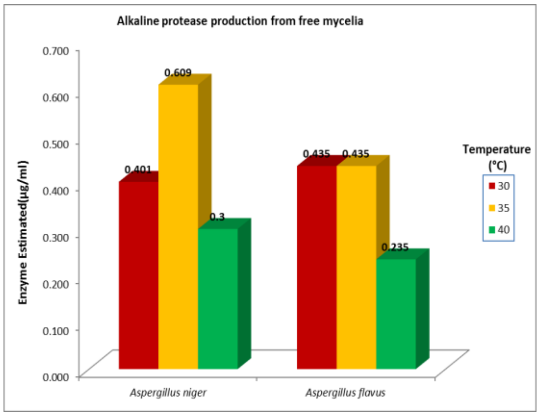

Figure 14

Effect of Temperature(free mycelia)

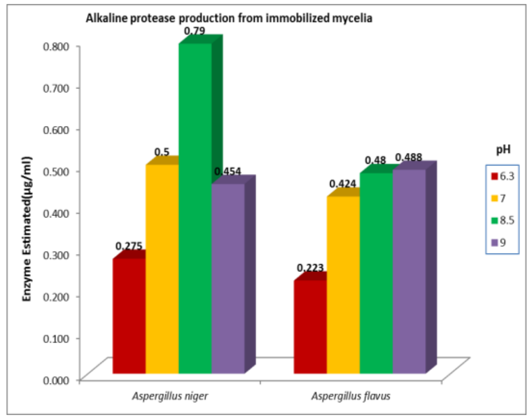

Figure 17

Effect of pH (Immobilized mycelia)

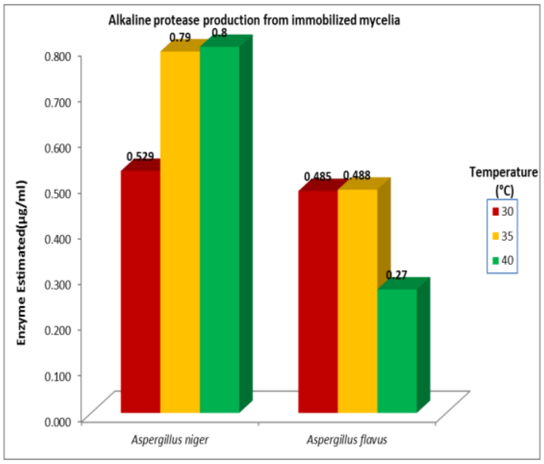

Figure 15

Effect of Temperature (immobilized mycelia)

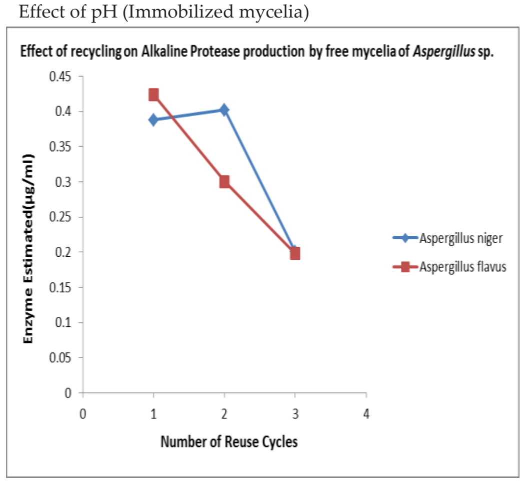

Figure 18

Effect of Recycling (free mycelia)

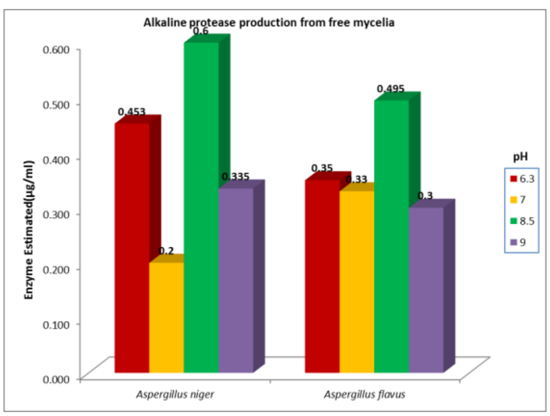

Figure 16

Effect of pH (free mycelia)

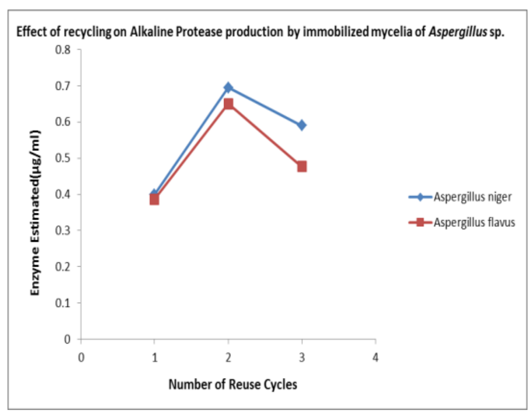

Figure 19

Effect of Recycling (immobilized mycelia)

4. CONCLUSION

Alkaline protease is an extracellular enzyme secreted in the production medium. This enzyme is produced by many fungal spp but the most widely used spps are *Aspergillus niger, Aspergillus oryzae, Aspergillus fumigatus* and *Aspergillus flavus* etc. From the work, it was found that *Aspergillus niger* and *Aspergillus flavus* were easily isolated from the soil sample and can withstand environmental fluctuations. These properties were further affected by the substrate concentration and other nutritional requirements, therefore to increase the withstanding property and the quantity or production of alkaline protease, immobilization technique was used. In this work, sodium-alginate was used as immobilizing matrix and resulted in enhanced production of protease enzyme.

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SUMMARY OF THE RESEARCH

1. Soil sample obtained from the waste dumping area showed more number of colonies of *Aspergillus niger* than any other fungi.
2. Production from immobilized mycelia was found to be more after two days when compared to free mycelia.
3. *Aspergillus niger* was found to produce more amount of protease than *Aspergillus flavus.*
4. Immobilized mycelia were found to withstand more environmental fluctuations.
5. Immobilized mycelia can be recycled.

FUTURE ISSUES

1. What is the rate of production of enzyme by bacteria under different environmental conditions?
2. How effectively the enzyme will work, when it is produced by the repeatedly used fungal mycelia in immobilization technique?
3. What are the alternative ways for the production of enzyme from fungus in large scale?

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Conflicts of interests

The authors declare that there are no conflicts of interests.

Data and materials availability

All data associated with this study are present in the paper.

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